

1. INTRODUCTION

Cancer, the second worldwide cause of death after cardiovascular diseases, is characterized by uncontrolled cell proliferation and the absence of cell death, which leads to the generation of an abnormal cell mass or tumor. While surgery and radiotherapy are the primary modes of treatment for local and nonmetastatic cancers, anticancer drugs are the current choice in the therapy of metastatic cancers.

Prostate cancer is a serious multidimensional disorder that arises because of a misrepresentation of the signaling cascades and acquired resistance against apoptosis. Prostate cancer is related to age, and is currently diagnosed with increasing incidence and prevalence rates as a consequence of the present increase in life expectancy and the availability of better diagnostic techniques. Prostate cancer has become the most common noncutaneous malignant disease in men in the EU. The mortality rate of prostate cancer is increasing disproportionately with the aging of the male population within the overall population growth, though the explanation of this alarming trend is still unavailable.

The morbidity and mortality rates of lymphoproliferative diseases are significant even currently, as 450,000 new cases and 225,000 deaths due to lymphoma are reported worldwide annually. These diseases are quite heterogeneous; thus, besides the classical morphological histology, immunophenotyping, the identification of cytogenetic, molecular characteristics and an overall assessment of the clinical features are essential for establishment of the exact diagnosis. Nevertheless, novel and highly precise molecular biological techniques have been developed and have become available recently for laboratories in hematopathology and molecular genetics departments. Among others, these techniques include the microarray techniques and the next-generation sequencing procedure, which can demonstrate the complexity and/or heterogeneity of lymphomas and may promote an understanding of the development of certain lymphoma subtypes.

Colorectal cancer is the third most common type of cancer and the fourth most common cause of cancer deaths globally, resulting in approximately 1.2 million new cases of cancer morbidity and 600,000 cases of mortality annually. The incidence is low under the age of 50 years, but it increases markedly with age. The average age at diagnosis in the developed countries is about 70 years. The highest incidences are reported in Europe, North America and Oceania.

The drug resistance most probably arises through a selection process, with the emergence of dominant clones from pools of heterogeneous tumor cells. For most chemotherapeutic agents, drug resistance can be stimulated by repeated treatment in tumor cell cultures. The main mechanisms of drug resistance of tumor cells are a decrease in drug accumulation, drug resistance mediated by detoxification of the drug in the cell, alterations of drug targets or through enhancement of target repair, and alterations in genes controlling apoptosis. One of the most important drug resistance mechanisms of tumor cells is the decrease of drug

accumulation by membrane-localized transporters. More than 200 proteins are involved in the transport of substrates across biological membranes, as members of the ABC (ATP-binding cassette) superfamily of proteins. Multidrug resistance (MDR) family member protein ABC transporters are transmembrane glycoproteins that promote the unidirectional translocation of structurally unrelated compounds via membrane barriers, utilizing the energy of adenosine triphosphate (ATP) hydrolysis. The ABC transporter superfamily is one of the largest and oldest protein families with conserved structural features and mechanism of action, preserved from prokaryotes to humans. MDR-ABC proteins expel a wide variety of substrates from the cells, including both endobiotics and xenobiotics. The MDR of tumor cells is frequently associated with overexpression of the membrane-localized ABC transporter B 1 subfamily (ABCB1; P-glycoprotein) encoded by the *ABCB1*-gene. ABCB1 demonstrates high transport capacity and a wide range of substrate specificity. These transported molecules are generally hydrophobic and amphipathic, uncharged or basic, though negatively charged molecules can also be transported. It has been established that ABCB1 is capable of interacting with more than 200 compounds, which can be classified on the basis of the transported substrates and the modulators. The most common approach through which to interfere with the overexpressed ABCB1 activity is the use of inhibitors. To date, three generations of ABCB1 inhibitors have been identified and generated, which can also be classified as competitive and noncompetitive inhibitors. As the name implies, the competitive inhibitors compete with the cytotoxic agents for transport. If they are successful, the cytotoxic agent could be transported by the pump, remaining inside the cell. Their noncompetitive counterparts do not compete for the same target. They bind to another target to change the pump conformation, so that the active site is no longer recognized by the substrates. Modulation of efflux pump-related mechanisms, in order to increase the activity of existing chemotherapeutics to which cancer becomes resistant, is one of the possible ways to overcome resistance.

2. AIMS OF THE STUDY

The primary aim of our study was to examine new perspectives of chemotherapy in connection with modification of the MDR of cancer. We set out to attempt to find a nontoxic and effective MDR-reversal agent among substituted steroids and phenothiazines. The activities of selected MDR-reversal compounds were studied in combination with anticancer chemotherapeutics in different cancer models *in vitro*. Apoptosis induction and inhibition of tumor promotion were studied in mouse T-lymphoma and prostate cancer models.

- Study of the antiproliferative effects of potential resistant modifiers by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assays on the L5178 mouse T-lymphoma parental cell line (PAR) and its L5178Y human *ABCB1*-gene transfected subline (MDR), LNCaP and PC3 prostate cancer cell lines and on the multidrug resistant Colo 320 colon adenocarcinoma cancer cell line

- Study of the cytotoxic effects of potential resistant modifiers by MTT assays on the PAR and MDR mouse T-lymphoma cell lines
- Study of the reversal of MDR by steroid and thioridazine derivatives on ABCB1-related resistance by flow cytometry, using the rhodamine 123 (R123) accumulation assay on the MDR mouse T-lymphoma cell line
- Study of the interactions of anticancer drugs with steroid and *N*-hydroxyalkyl-2-aminophenothiazine resistance modifiers, using an *in vitro* model for combination chemotherapy by a checkerboard microplate method in the PC3 and multidrug resistant colon adenocarcinoma Colo 320 cells, respectively
- Study of the apoptosis induction of the selected steroid and thioridazine resistance modifiers measured by Annexin V-fluorescein isothiocyanate (FITC) assay, using flow cytometry in the MDR mouse T-lymphoma and PC3 prostate cancer cell lines
- The evaluation of structure-activity relationships of steroid derivatives, with docking calculations based upon biological studies
- Study of the role of thioridazine stereoisomers in anticancer activity

3. MATERIALS AND METHODS

3.1. Compounds

- Steroid derivatives

Aminosteroids **14** and **15** were prepared from the oximes of the corresponding ketosteroids by sodium tetrahydroborate reduction in the presence of NiCl₂. The aminosteroids obtained were acylated with *N*-*tert*-butyloxycarbonyl (BOC)-protected amino acids by a mixed anhydride method (**1–5**), and the protecting group was eliminated with dry hydrogen chloride in dioxane solution, affording amine hydrochlorides **6–12**. Alkyl-aminomethylene steroids were prepared from 16-hydroxymethylene-17-ketosteroids and primary amines, and were acetylated with acetic anhydride (**16–20**). The D-condensed heterocyclic steroid **21** was prepared from 16-hydroxymethylene-3-methoxyestra-1,3,5(10)-trien-17-one with guanidine. The 16-aminomethylandrosterone derivative **13** was synthesized from the corresponding 16-methylene-17-ketone by the addition of *n*-propylamine and selective *O,O*-desacetylation of the fully acetylated product. Samples of the above-mentioned compounds were dissolved in DMSO as 2.0 mg/ml stock solutions. Verapamil as positive control, DMSO and *N*-BOC-isoleucine-*O*-pentachlorophenyl ester (**23**) were obtained from Sigma–Aldrich (St. Louis, MO, USA).

- Phenothiazine derivatives

a) Thioridazine hydrochloride is a racemic mixture of enantiomers with an asymmetric carbon at position 2 in the piperidyl ring. The racemic thioridazine was purchased from Sigma–Aldrich, Vallensbak Strand, Denmark. Thioridazine in its ordinarily

clinically prescribed form is a racemic mixture of equal amounts of the (+) and (–) enantiomers. The enantiomers were separated by resolution of the commercially available thioridazine racemate.

- b) The 26 tested *N*-hydroxyalkyl-2-aminophenothiazine derivatives were prepared by recently elaborated chemical transformations. Thus, derivatives **1a–o** were obtained by protection and Buchwald–Hartwig amination of **1**, R = Cl, whereas **2** was obtained as a by-product of the hydroboration-oxidation transformation of the appropriately substituted dienyphenothiazine. Sulfoxides **3a–e** and sulfones **3f–j** were prepared by oxidation of the related phenothiazine **1** with *m*-chloroperoxybenzoic acid (*m*-CPBA). The *N*-hydroxyalkyl-2-aminophenothiazine derivatives were dissolved in DMSO.

3.2. Cell cultures

- L5178 mouse T-cell lymphoma cells (PAR) (ECACC Cat. No. 87111908) were transfected with pHa MDR1/A retrovirus and the *ABCB1*-expressing cell line L5178Y (MDR).
- Prostate cancer cell lines PC3 (ATCC® Cat. No. CRL-1435) and LNCaP (ATCC® Cat. No. CRL-1740) were used.
- Human colon adenocarcinoma cell lines the Colo 205 doxorubicin sensitive parent and Colo 320/MDR-LRP doxorubicin resistant expressing *ABCB1* (MDR1-LRP), ATCC® Cat. No. CCL-220.1 (Colo 320) and ATCC® Cat. No. CCL-222 (Colo 205) were applied.

3.3. Assays for antiproliferative effect

The effects of increasing concentrations of compounds on cell growth were determined in 96-well flat-bottomed microtiter plates. The compounds were serially diluted in 100 μ L of McCoy's 5A or RPMI-1640 medium, respectively. 6×10^3 mouse T-cell lymphoma cells (PAR or MDR), 5×10^3 human colonic adenocarcinoma cells (Colo 205 or Colo 320) in 50 μ L of medium or 1×10^4 PC3 prostate cancer cells in 100 μ L of medium were then added to each well, with the exception of the medium control wells. The culture plates were further incubated at 37 °C under 5% CO₂ for 72 h. At the end of the incubation period, 15 or 20 μ L of MTT solution depending on the final volume (from a 5 mg/mL stock) was added to each well and, after another 4 h, 100 μ L of 10% sodium dodecylsulfate (SDS) in 0.01 N HCl was measured into each well. The culture plates were further incubated at 37 °C overnight. The cell growth was determined by measuring the optical density (OD) at 540 nm (ref. 630 nm) with a Multiscan EX ELISA reader (Thermo Labsystem, Cheshire, WA, USA). In the assay, the solvent did not have any effect on the cell growth at the concentrations used for half-maximal inhibitory concentration (IC₅₀) calculations. IC₅₀ values and the standard error of the

mean (SEM) of triplicate experiments were calculated by using GraphPad Prism software version 5.00 for Windows with nonlinear regression curve fit (GraphPad Software, San Diego, CA, USA; www.graphpad.com).

3.4. Assays for cytotoxic effect

The effects of increasing concentrations of the drugs alone on cell growth were tested in 96-well flat-bottomed microtiter plates. The compounds were diluted in a volume of 100 μL medium. 2×10^4 mouse T-cell lymphoma cells (PAR or MDR) in 50 μL of medium were then added to each well, with the exception of the medium control wells. The culture plates were further incubated at 37 $^{\circ}\text{C}$ for 24 h; at the end of the incubation period, 15 μL of MTT solution (from a 5 mg/mL stock) was added to each well. After incubation at 37 $^{\circ}\text{C}$ for 4 h, 100 μL of SDS solution (10% in 0.01 M HCl) was added to each well, and the plates were further incubated at 37 $^{\circ}\text{C}$ overnight. The cell growth was determined by measuring the OD at 540 nm (ref. 630 nm) with a Multiscan EX ELISA reader (Thermo Labsystems, Cheshire, WA, USA). In the assay, the solvent did not have any effect on the cell growth at the concentrations used for IC_{50} calculations. IC_{50} values and the SEM of triplicate experiments were calculated by using GraphPad Prism software version 5.00 for Windows with nonlinear regression curve fitting (GraphPad Software, San Diego, CA, USA; www.graphpad.com).

3.5. Fluorescence uptake assays

The cell numbers of the L5178 MDR and L5178Y PAR cell lines were adjusted to 2×10^6 cells/mL, re-suspended in serum-free McCoy's 5A medium and distributed in 0.5 mL aliquots into Eppendorf centrifuge tubes. Steroid compounds were added at a final concentration of 2 $\mu\text{g}/\text{mL}$ and thioridazine and its enantiomers were added at a final concentration of 0.25 and 2.5 $\mu\text{g}/\text{mL}$, and the samples were incubated for 10 min at room temperature. Verapamil was applied as a positive control at 10 $\mu\text{g}/\text{mL}$. DMSO was added to the negative control tubes at the same volume as used for the tested compounds. No activity of DMSO was observed. Next, 10 μL (5.2 μM final concentration) of the fluorochrome and ABCB1 substrate R123 was added to the samples, and the cells were incubated for a further 20 min at 37 $^{\circ}\text{C}$, then washed twice and re-suspended in 0.5 mL of PBS for analysis. The fluorescence of the cell population was measured with a PartecCyFlow[®] flow cytometer (Partec, Münster, Germany). The percentage of the mean fluorescence intensity was calculated for the treated MDR cells as compared with the untreated cells. A fluorescence activity ratio (FAR) was calculated via the following equation on the basis of the measured fluorescence values:

$$\text{FAR} = \frac{\text{MDR}_{\text{treated}}/\text{MDR}_{\text{control}}}{\text{parental}_{\text{treated}}/\text{parental}_{\text{control}}}$$

3.6. Apoptosis assays

The capacity of the compounds to induce apoptosis was investigated by using the MDR mouse T-lymphoma cell line and the human PC3 prostate cancer cell line. The cell number was adjusted to 5×10^5 cells/mL and this material was distributed in 0.5 mL aliquots into 24-well plates. The apoptosis inducer 12*H*-benzo[α]phenothiazine (M627) was used as positive control at 25 μ g/mL. Compounds were tested at 4 μ g/mL. Wells containing no M627 or steroidal compounds served as negative controls. The cells were incubated at 37 °C for 1 h, and at the end of the incubation period the culture medium was removed, the cells were washed with PBS, and 0.5 mL of fresh culture medium was added to the cells. Samples were transferred to 24-well culture plates and further incubated overnight at 37 °C, under 5% CO₂. The apoptotic activities of the compounds were evaluated by using the Annexin-V FITC Apoptosis Detection Kit (Cat. No. PF 032 from Calbiochem) in accordance with the manufacturer's instructions. The fluorescence of the cell population was analyzed immediately with a Partec CyFlow[®] flow cytometer (Partec, Münster, Germany). In case of PC3 cells, the cells were seeded and incubated overnight at 37 °C, 5% CO₂. After the treatment the same protocol was used as described above.

3.7. Checkerboard combination assays

A checkerboard microplate method was applied to study the effects of the drug interactions between the *N*-hydroxyalkyl-2-aminophenothiazine derivatives and the chemotherapeutic drug doxorubicin on MDR colon adenocarcinoma cells overexpressing the ABCB1 transporter. The drug interactions between the substituted steroid compounds and doxorubicin were also investigated on the PC3 prostate cancer cell line. The dilutions of doxorubicin were made in a horizontal direction in 100 μ L, and the dilutions of the *N*-hydroxyalkyl-2-aminophenothiazines or steroid compounds were made vertically in the microtiter plate in a volume of 50 μ L. The cells were re-suspended in culture medium and distributed into each well in 50 μ L portions containing 1×10^4 Colo 320 cells or 6×10^3 PC3 prostate cancer cells. The plates were incubated for 72 h at 37 °C in a CO₂ incubator. The cell growth rate was determined after MTT staining, as described above. The combination index (CI) values at 50% growth inhibition (ED₅₀) were determined by using CompuSyn software to plot 4 or 5 data points for each ratio. CI values were calculated by means of the median-effect equation, where CI < 1, CI = 1 and CI > 1 represent synergism, an additive effect (or no interaction) and antagonism, respectively.

3.8. Molecular docking

2D structures of the chemical entities were drawn and later energy-minimized into 3D structures by using Corina Online Demo. All 3D structures were saved in PDB format ready to be docked. Molecular docking was conducted following a protocol previously reported by Zhao et al.

4. RESULTS

4.1. Antiproliferative effects of potential resistant modifiers

Twenty-three substituted steroid derivatives were synthesized, and their antiproliferative effects on the PAR, MDR, LNCaP and PC3 cell lines were studied. Evaluation of the antiproliferative activities of the compounds revealed that **8** and **10–14** were the most active against the parental mouse T-lymphoma cell line, with IC₅₀ values of < 5 µg/mL. With the exceptions of **9** and **13**, all of the above-mentioned compounds and **22** were also active against the MDR cell line. As concerns the two different prostate cancer cell lines, the most effective compounds were **3**, **12**, **18**, **19** and **23** against the LNCaP cells, and **12** against the PC3 cells. Thus, **12** was the only compound which exhibited strong activity against all four studied cell lines.

The *N*-hydroxyalkyl-2-aminophenothiazine derivatives displayed more potent cytotoxic effects on the resistant colon adenocarcinoma cell than on the sensitive cell line Colo 205. The IC₅₀ values of the derivatives on the sensitive cell line Colo 205 can be divided into four groups: those with an IC₅₀ of > 100 µM: **1i**, **3f**, **3g**, **3h** and **3i**; those with an IC₅₀ in the interval 20–55 µM: **1a**, **1b**, **1d**, **1f**, **1g**, **1l**, **1n**, **3a**, **3b**, **3c** and **3e**; those with an IC₅₀ in the interval 10–19 µM: **1c**, **1j**, **1m**, **2**, **3d** and **3j**; and those with an IC₅₀ in the interval 5–9 µM: **1e**, **1h** and **1k**. The compounds were more selective for the MDR Colo 320 cells, as shown by their lower IC₅₀ values. In this case, the compounds can be divided into three categories: those with an IC₅₀ in the interval 10–40 µM: **1a**, **1b**, **1d**, **1e**, **1f**, **1g**, **1i**, **1l** and **3a**; those with an IC₅₀ in the interval 3–10 µM: **1c**, **1h**, **1j**, **1m**, **1n**, **2**, **3c**, **3d**, **3e**, **3f**, **3i** and **3j**; and those with an IC₅₀ of < 3 µM: **1k**, **1o**, **3b**, **3g** and **3h**.

No significant differences were found between the racemate and the (+) and (–) enantiomers of thioridazine as concerns their antiproliferative activity against the MDR mouse lymphoma cell line.

4.2. Cytotoxic effects of potential resistance modifiers

It can be concluded that the steroid derivatives possess slight cytotoxic effects, although there was no significant difference between the parental and the MDR mouse T-lymphoma cells. The most active derivatives were **4**, **9** and **12**.

4.3. Reversal of multidrug resistance on mouse lymphoma

Twenty-three modified steroid derivatives were examined on MDR cells in R123 excluding short-term experiments at subinhibitory concentrations for 30 min. In this experiment, 2×10⁶ cells were exposed to 2 µg/mL final concentrations for 10 min before indicator R123 was added to the cells. The aminosteroid compounds acylated with BOC-amino acids (**1–5** and **9**) and even the simple *N*-acetyl derivatives (**13**, **16**, **18** and **19**) displayed pronounced activity in the reversal of the MDR of the MDR mouse T-lymphoma cell line, with FAR values between

29 and 77. Steroids containing free amino groups or aminehydrochloride substituents demonstrated weaker or no activity on MDR reversal as measured by the accumulation of R123. Interestingly, **12** exhibited only mild inhibition at the concentration used. As concerns the activities expressed in μM , the most active compound was **18**, with an FAR of 77.41 at 4.4 μM . **16**, **13**, **1**, **19** and **3** were also highly active.

The effects of the racemic and the (+) and (–) enantiomers of thioridazine on the retention of R123 in the MDR mouse lymphoma cell line were studied at 0.25 and 2.5 $\mu\text{g}/\text{mL}$. At 0.25 $\mu\text{g}/\text{mL}$, thioridazine and its enantiomers moderately inhibited the ABCB1 expressed by the MDR mouse T-cell lymphoma cell line, while at 2.5 $\mu\text{g}/\text{mL}$ the inhibition was stronger, without apparent stereospecificity, which appears to be a promising finding.

4.4. *In vitro* combinations (checkerboard assays)

The effects of the substituted steroid compounds on the activity of doxorubicin were observed to vary from ineffective to strong synergy. Five compounds gave rise to strong synergy with doxorubicin: **4**, **9**, **10**, **14** and **22**. Moderate synergy was observed with **1**, **2**, **8**, **12**, **16**, **17**, **19** and **23**, while **3** and **18** were ineffective. The IC_{50} values of **5–7**, **11**, **13**, **15**, **20** and **21** could not be calculated, and the interactions of these compounds with doxorubicin could therefore not be determined.

With regard to the ABCB1-modulating activities of the lead *N*-hydroxyalkyl-2-aminophenothiazine compounds, six derivatives were selected for combination studies with doxorubicin. All of them exhibited synergistic activity with doxorubicin in Colo 320 cells at an *N*-hydroxyalkyl-2-aminophenothiazine derivative:doxorubicin ratio of 12:1. It can be concluded that the lead compound of the *N*-hydroxyalkyl-2-aminophenothiazine series was **3j**, which showed synergism with the anticancer drug doxorubicin on MDR colon adenocarcinoma cells.

4.5. *Apoptosis-inducing effects of selected steroid compounds and thioridazine*

The apoptosis-inducing effects of the substituted steroid compounds on the PC3 cell line were measured at the nontoxic concentration of 4 $\mu\text{g}/\text{mL}$. These compounds proved to exert no relevant apoptosis-inducing activity: the proportion of early apoptosis was 1–6%, those of late apoptosis and necrosis were 1–5%, and that of cell death was around 1%. No significant difference was seen when these data were compared with those on the untreated control.

The apoptosis induction assays were carried out at 2.5 and 5 $\mu\text{g}/\text{mL}$ racemic thioridazine and its enantiomers in MDR mouse T-lymphoma cells. Racemic thioridazine and the enantiomers displayed similar apoptosis-inducing activities, suggesting that stereoselectivity does not play a role in the induction of apoptosis. Both racemic thioridazine and the enantiomers induced early apoptosis in PC3 cells, but there was no significant difference between them. Our results clearly demonstrated that racemic thioridazine and its enantiomers have essentially the same activity against cancer cell lines with respect to the inhibition of replication and the induction of apoptosis.

4.6. Molecular docking

Molecular docking is an effective computational technique which provides an estimate of the binding energy of a compound to the target protein. A scoring system is used to detect the ideal docking configuration. The amino acids involved in hydrophobic and hydrogen-bonding interactions are also predicted by the algorithm. Our molecular docking results suggested that the tested compounds inhibit ABCB1 protein (P-glycoprotein) activity through binding to the drug-binding pocket, which is the same binding site as that for verapamil. We carried out *in silico* investigations of the interaction of the panel of steroid compounds with P-glycoprotein, using verapamil as a positive control. All 23 compounds exhibited binding energies lower than that of verapamil. The binding energies ranged from -6.43 to -9.88 kcal/mol. The predicted inhibition constants ranged from 0.1 to 10.1 μM .

5. DISCUSSION

Substituted steroids **8**, **10–12**, **14** and **22** exerted effective antiproliferative activity against MDR mouse T-lymphoma cells expressing ABCB1. These compounds are aminoacylamide salts based on three different steroid skeletons (androstane, androstene and pregnandiene). **22** is an estrone ether derivative, and has a bidentate α -hydroxymethylene-ketone arrangement on the D-ring, allowing the possibility of a 1,4-hydrogen donor–acceptor connection. On the other hand, **3**, **12**, **18**, **19** and **23** proved to be effective in the antiproliferative assays against the LNCaP prostate cell line. In the MDR reversal studies, a majority of the investigated compounds were effective: **1–5**, **9**, **13** and **16–20**. In contrast, **6**, **8**, **10–12**, **14**, **15** and **21** had only weak effects. These latter molecules are either amine hydrochlorides or possess special structures; **14**, **15**, and **21** are estrone ether derivatives. Aminosteroids with basic primary amino groups had practically no effect. Our molecular docking results suggested that the tested compounds inhibit ABCB1 activity through binding to the drug-binding pocket, which is the same binding site as for verapamil. Most of the compounds with high activity and verapamil share one or more amino acids in their binding sites. It appears that there is a significant correlation between the binding energies of the compounds and their FARs. A large number of the compounds were able to enhance the activity of doxorubicin against the PC3 cell line in combination experiments. On the basis of our results, **9** could be a lead compound for further investigations relating to combined chemotherapy, while **12** might be a promising candidate as an effective antiproliferative chemotherapeutic agent.

The investigated *N*-hydroxyalkyl-2-aminophenothiazines exert promising antiproliferative effects on MDR colonic adenocarcinoma cells. The nature of the substituents can strongly influence the anticancer activity of the derivatives. From our understanding of the relationship between structural diversity and activity, we concluded that the compounds substituted with secondary amines (morpholine, diethylamine or *N*-methylpiperazine) at position 2 in the *N*-hydroxyalkyl-2-aminophenothiazine ring (**3c**, **3d** and **3f**) were the most selective candidates.

Further, the IC₅₀ data revealed that the oxidized form of the sulfur atom (sulfoxide or sulfone) led to marked selectivity (**3h**, **3i** and **3j**). The compounds containing a primary amine or acid amide at position 2 in the ring, such as **1c**, **1h**, **1j**, **1m** and **1n**, displayed lower activities as compared with those of the aforementioned molecules. It can be assumed that the modulation of similar structural details (secondary amines at position 2, a 2-hydroxy group in the alkyl chain, sulfoxide or sulfone) of the chosen lead compounds (**1c**, **3d**, **3g**, **3h**, **3i** and **3j**) is responsible for the synergism with doxorubicin. The antiproliferative activity findings clearly demonstrate that the oxidation of the sulfur atom in **1h** resulted in the lead molecule **3j**. It was also concluded that this structural modification increased the biological activity.

The importance of stereochemistry in drug action is gaining ever-greater attention in medical practice. However, our results clearly indicate that the racemate thioridazine and its enantiomers have essentially the same activity against MDR mouse T-lymphoma and PC3 prostate cancer cell lines with respect to the inhibition of replication, the induction of apoptosis and the inhibition of ABCB1.

6. NEW FINDINGS

- Aminoacylamide salts on the steroid skeleton exerted pronounced antiproliferative activity against MDR mouse T-lymphoma and LNCaP prostate cancer cell lines.
- Aminosteroids with basic primary amino groups had no anticancer effect in prostate cancer cell lines.
- No significant differences were found between the racemate and the (+) and (–) enantiomers of thioridazine as concerns antiproliferative activity against the MDR mouse T-lymphoma cell line.
- The separate use of one individual enantiomer does not appear to provide any advantage over use of the racemic mixture of thioridazine for the adjuvant therapy of MDR cancer.
- *N*-hydroxyalkyl-2-aminophenothiazines substituted with secondary amines at position 2 in the ring and the oxidation state of the sulfur atom (sulfoxide or sulfone) influence the antiproliferative activity on MDR colonic adenocarcinoma cells.
- A large number of substituted steroid compounds were able to enhance the activity of doxorubicin against the PC3 cell line in combination experiments.
- *N*-hydroxyalkyl-2-aminophenothiazines containing secondary amines at position 2, a 2-hydroxy group in the alkyl chain, or sulfoxide or sulfone displayed synergistic effects with doxorubicin.
- None of the substituted steroid derivatives or thioridazine compounds induced marked apoptosis either in PC3 prostate cancer cells or in MDR mouse T-lymphoma cells.
- Molecular docking is an effective computational technique which estimates the binding energy of a compound to the target protein. The tested steroid compounds proved to inhibit ABCB1 activity through binding to the drug-binding pocket, which is the same binding site as for verapamil.

7. ACKNOWLEDGMENTS

I would like to express my deep and sincere gratitude to my supervisor, **Prof. Dr. József Molnár**. He raised my interest in the experimental work, and gave me the pleasure of success. He understood my clinical problems and helped me to put them into a laboratory setting, and to find solutions. Through his open-mindedness, he has provided me with excellent support.

I would also like to express my grateful thanks to **Prof. Dr. Endre Varga** and **Prof. Dr. Aurél János Simonka**, present and past chairs of the Department of Traumatology, who ensured the conditions for my research work beside my clinical work.

I would further like to express my sincere gratitude to **Dr. Katalin Burián** and **Prof. Dr. Yvette Mándi**, present and past chairs of the Department of Medical Microbiology and Immunobiology, for providing me with the possibility to work at the department.

My special thanks are due to **Dr. Gabriella Spengler**, **Mrs. Anikó Vigyikánné Váradi**, **Dr. Ana Martins** and **Prof. Dr. Leonard Amaral** at the Department of Medical Microbiology and Immunobiology. I wish to thank them for all their help and support and for the interest they have shown.

I am likewise grateful to **Dr. István Gárgyán** and **Dr. Gábor Süveges**, senior consultants, for their support in my clinical work.

I would like to thank **Dr. Imre Ocsovszki** for performing the flow-cytometric measurements.

I feel a deep sense of gratitude to my wife and my family for always being so ready to help.

I express my thanks to all my co-workers, colleagues and staff members at the Departments of Microbiology and Traumatology, for creating a supportive and pleasant working environment.

I dedicate my thesis to my patients.

8. FINANCIAL SUPPORT

The work on which this thesis was based was supported by the following organizations and grants:

- TÁMOP 4.2.4.B/2-11-1-2012-0001 (Campus Hungary Programme)
- TÁMOP 4.2.2.A-11/1/KONV-2012-0035
- Szeged Foundation for Cancer Research

9. PUBLICATIONS

1. Csonka Á, Spengler G, Martins A, Ocsovszki I, Christensen JB, Hendricks O, Kristiansen JE, Amaral L, Molnár J: Effect of thioridazine stereoisomers on the drug accumulation of mouse lymphoma and human prostate cancer cell lines *in vitro*. In Vivo 27(6): 815–820, 2013. **IF:1.148**
2. Csonka Á, Hamdoun S, Spengler G, Martins A, Vincze I, Efferth T, Molnár J: Substituted steroidal compounds containing amino- and amido groups reverse multidrug resistance of mouse T-lymphoma and two human prostate cancer cell lines *in vitro*. Anticancer Research 35(4): 2105–2112, 2015. **IF:1.872**
3. Takács D, Csonka Á, Horváth Á, Windt T, Gajdács M, Riedl Zs, Hajós Gy, Amaral L, Molnár J, Spengler G: Reversal of ABCB1 related multidrug resistance of colon adenocarcinoma cells by phenothiazines. Anticancer Research 35(6): 3245–3252, 2015. **IF:1.872**